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Rapid Detection of Bacterial Endotoxins in Drinking Water and Renovated Wastewater

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A pilot study was conducted to determine the feasibility of using the *Limulus* endotoxin assay to detect endotoxins in potable waters and from reclaimed advanced waste treatment (AWT) plant effluents. Water samples were tested using both *Limulus* lysates prepared in our laboratory and a commercial product, Difco Pyrotest. The *Limulus* assay procedure was easily adapted to the testing of water samples for endotoxin. Measured endotoxin concentrations varied from 0.78 ng/ml to 1,250 ng/ml. Levels of endotoxin were not predictable based on whether the water was drinking water or AWT water, i.e., some AWT water samples had less endotoxin activity than some samples of drinking water, and some AWT waters had greater endotoxin activity than drinking water. Only three of the water samples tested were free of any detectable endotoxin. Breakpoint chlorination procedures seemed to reduce measurable endotoxin content, whereas passage through activated carbon columns was associated with greater final endotoxin concentrations in test waters.

It is well recognized that increased urban and industrial requirements for water may make use of renovated wastewater a necessity. The Health Effects Research Laboratory of the U.S. Environmental Protection Agency (USEPA) is interested in the public health significance of biological materials that may be present in effluents from advanced waste treatment (AWT) facilities, so that appropriate treatment measures may be designed now for removal of potentially harmful materials. Previous efforts at controlling and eliminating toxic pollutants have been directed at those known to be present initially. However, many potential pollutants are not well known, including a host of biological degradation and metabolic by-products which are created in the treatment process. Prominent among these are pyrogens, notably bacterial endotoxin produced by gram-negative bacteria.

Levin and Bang originally described an endotoxin bioassay based on the coagulation of a lysate prepared from the amebocytes of the horseshoe crab, *Limulus polyphemus* (10). The *Limulus* assay can easily detect as little as 1 ng of bacterial endotoxin per ml of fluid after an assay period of less than 2 h. This assay is now recognized as the most sensitive test available for the detection of bacterial endotoxin (1, 14). The test is simple, specific, rapid, and inexpensive compared to the USP rabbit pyrogenicity test (1). Since its introduction, the *Limulus* assay has been applied to the detection of endotoxin in a variety of fluids, including blood from patients suspected of gram-negative sepsis (11, 16), for the study of experimental endotoxemia and shock in laboratory animals (2, 3), for rapid detection of gram-negative bacterial meningitis (13, 15), for screening of urine for significant bacteriuria (5, 6), and as a method for detection of pyrogenic parenteral fluids (1, 8). A previous report by DiLuzio and Friedmann (4) has suggested that the *Limulus* assay might also be used for the detection of bacterial endotoxin in drinking water and other surface waters.

The present study was undertaken to determine the feasibility of the *Limulus* assay to measure endotoxins in actual or potential drinking waters and highly treated waste waters.

MATERIALS AND METHODS

Water samples. Twenty-five water samples were supplied for *Limulus* testing by the USEPA. These included both normal drinking waters from several cities in the United States and AWT process sample from active AWT plants. Certain AWT water samples were dechlorinated by the addition of 0.1 ml of 2% sodium thiosulfate prior to shipment. Sodium thiosulfate was not added to any of the potable water samples.

Mailing and storage of water samples. Water samples were mailed from various locales using sterile, pyrogen-free, plastic test tubes (Falcon) and a composite mailing container. Certain water samples were frozen at their site of origin prior to being mailed. Samples were received in the laboratory in a cool but not frozen condition. Upon receipt in the laboratory, all water samples were refrigerated until testing.

***Limulus* lysate.** Two sources of *Limulus* amebocyte lysate were used for this study. Lysate was prepared from horseshoe crabs in our laboratory by methods previously described (7), and a commercial source of ameobycyte lysate, Difco Pyrotest, obtained from Difco Laboratories (Detroit, Mich.) was also used. The sensitivities of the lysates prepared in our laboratory ranged from 0.39 to 2.5 ng of *Escherichia coli* endotoxin per ml. Four different lots of Difco Pyrotest were evaluated. Three of these lots could detect as little as 0.625 ng/ml, and the fourth lot could detect as little as 1.25 ng of the same *E. coli* endotoxin per ml.

Performance of *Limulus* assay. An acceptable pH range for testing with the *Limulus* assay was considered to be between pH 5.5 and 7.5. Two water samples were received with a pH of 11.2, requiring adjustment with a standard acid solution (1 N HCl). When tests were performed using our lysate preparation, water samples were assayed by simply adding 0.1 ml of the untreated water to 0.1 ml of *Limulus* lysate in disposable pyrogen-free glass test tubes (10 by 75 mm). Tests using Pyrotest were performed by adding 0.2 ml of the untreated water sample directly to the single test vial containing lyophilized *Limulus* lysate. All *Limulus* assays were incubated for 70 to 90 min at 37°C. After incubation, the presence of a gel or a marked increase in viscosity and turbidity was considered a positive test for endotoxin. Test results were also graded from 1+ to 4+ using criteria previously described (7).

Quantification of endotoxin. The levels of endotoxin activity in water samples were determined by dilution of samples in a pyrogen-free diluent. Dilutions were prepared in pyrogen-free 0.9% NaCl (Travenol Laboratories) for testing with our amebocyte lysate; pyrogen-free distilled water (Travenol Laboratories) was used as the diluent for tests performed using Pyrotest. For purposes of discussion, endotoxin activity was reported in endotoxin equivalents. One endotoxin equivalent is defined as the activity of an unknown specimen that is equivalent to the activity produced by a saline or water suspension of a standard endotoxin, in this case chemically purified *E. coli* endotoxin (supplied by Difco Laboratories), after incubation with an equal volume of *Limulus* amebocyte lysate at 37°C for 1 to 2 h (12). The number of endotoxin equivalents present in a sample was calculated by comparing its activity with endotoxin standards tested with the same lot of *Limulus* lysate. The level of endotoxin present in a given sample, therefore, was the highest dilution of that sample giving a positive *Limulus* test, multiplied by the sensitivity of the lysate used, e.g., 1:20 sample dilution × 0.625-ng/ml endotoxin sensitivity = 12.5 ng of endotoxin equivalents per ml in the water sample.

***Limulus* assay inhibition studies.** The possibility of inhibition or a decrease in the sensitivity of the *Limulus* test due to interfering substances in water was determined. This was accomplished by "spiking" water samples with known amounts of endotoxin to obtain an endotoxin solution of known concentration prepared in a particular water sample. These spiked water samples were then tested in parallel with endotoxin solutions prepared in known, noninhibitory, pyrogen-free water or saline (Travenol). Any loss of reactivity due to the test solvent was thus determined.

RESULTS

The *Limulus* test was found to be easily and rapidly adapted to the testing of water samples for endotoxin without any modification of the *Limulus* assay procedure. Some inhibition was seen when testing undiluted water samples with our lysate. However, this problem of inhibition was easily alleviated by diluting the water sample in pyrogen-free 0.9% saline. A recently reported modification of the method for preparing *Limulus* amebocyte lysate is said to alleviate this apparent sodium requirement (17). Furthermore, no such inhibition was seen with the commercial *Limulus* amebocyte lysate, Difco Pyrotest. Using Pyrotest, water samples were easily tested by addition of untreated, undiluted water directly to the lyophilized lysate in a single test vial. By use of serial dilutions of the unknown water samples in pyrogen-free distilled water or saline, quantitation of endotoxin activity in the water samples could be easily achieved.

Table 1 shows the results of *Limulus* assays on samples from 10 public drinking water systems throughout the United States. The range of endotoxin concentrations varied widely; one water sample had no detectable endotoxin activity (Miami), whereas others had as much as 500 ng of endotoxin equivalents per ml.

Table 2 lists the results of AWT sample testing and the type of treatment used at the six pilot plants. All AWT effluent samples were frozen prior to mailing. The only samples which were free of detectable endotoxin were taken at the Escondido plant, which uses reverse osmosis as the final treatment step. In other plants, if activated carbon columns were used near the end of the treatment process, there were detectable amounts of endotoxin present. The only plant to use breakpoint chlorination following the carbon filters, the Blue Plains pilot plant, had relatively low endotoxin levels. Endotoxin levels from Pomona process 3 samples, where ozonation was used, did not differ substantially from processes 1 and 2, where normal chlorination was practiced.

The use of sodium thiosulfate to dechlorinate

TABLE 1. *Results of Limulus tests on drinking water samples*

Municipal water plant	Treatment systems	Typical free chlorine (mg/liter)[a]	Sample frozen	Endotoxin equivalents (ng/ml)
Cincinnati, Ohio	Prechlorination, sedimentation, coagulation-filtration, postchlorination	1.4	Yes	1.25
Grand Forks, Iowa	Prechlorination, coagulation-filtration, softening, powdered activated carbon, postchlorination	0	No	12.5
Seattle, Wash.	Chlorination	0	No	12.5
New York, N.Y	Chlorination	0.05	No	12.5
Miami, Fla.	Prechlorination, softening, sedimentation recarbonation, breakpoint chlorination, filter	2.1	No	<0.625
Boston, Mass.	Chlorination	0	Yes	500
Lawrence, Mass.	Prechlorination, granular-activated carbon, coagulation-filtration, postchlorination	0.8	Yes	125
Philadelphia, Pa.	Prechlorination, sedimentation, coagulation-filtration, postchlorination	1.5	No	10
Terrebonne Parish, La.	Prechlorination, granular-activated carbon, coagulation-filtration, postchlorination	0	No	2.5
Ottumwa, Iowa	Prechlorination, powdered-activated carbon, coagulation-filtration, postchlorination	1.9	No	2.5

[a] Free chlorine values are those obtained during the National Organics Reconnaissance Survey of 1975.

TABLE 2. *Results of Limulus tests on AWT water samples*

AWT plant	Treatment system	Sodium thiosulfate added	Endotoxin equivalents (ng/ml)
Blue Plains pilot plant, Washington, D.C.	Screening + lime + nitrifying activated sludge + denitrification + carbon + multimedia filter + breakpoint chlorination	Yes	2.5
		No	12.5
Lake Tahoe, Nev.	Activated sludge + lime + NH_3 strip + CO_2 + multimedia filter + carbon + chlorination	Yes	1,250
		No	1,250
Orange County, Calif.	Trickling filter + lime + settling	Yes	0.78
		No	0.78
Escondido, Calif.	Activated sludge + sand filter + reverse osmosis	Yes	<0.313
		No	<0.313
EPA AWT pilot plant, Cincinnati, Ohio	Trickling filter + settling + lime + flocculation + settling + CO_2 + multimedia filter + chlorination + carbon	No	125
Pomona, Calif., Process 1	Activated sludge + carbon + chlorination	Yes	125
		No	500
Pomona Process 2	Activated sludge + carbon + chlorination + carbon	Yes	250
		No	125
Pomona Process 3	Activated sludge + carbon + ozone + carbon	Yes	250
		No	250

the samples did not seem to affect the endotoxin results significantly. Sodium thiosulfate was not added to any of the potable water samples. Of the three samples that were frozen prior to air-mailing to the laboratory, one had the lowest and one the highest endotoxin value.

DISCUSSION

This preliminary investigation has demonstrated the feasibility of testing water samples for the presence of pyrogenic substances, namely gram-negative bacterial endotoxins by the *Limulus* assay. Previous research indicates that the *Limulus* test satisfies the criteria of an ideal in vitro laboratory procedure for detecting pyrogens. As a result, its use is currently being permitted by the Food and Drug Administration to determine possible endotoxin contamination of ingredients used to prepare parenteral and biological products and for in-line testing of these products in the pharmaceutical industry. In our hands, the *Limulus* assay has proven to be a simple, relatively inexpensive, sensitive, and reliable procedure for the assay of endotoxin-like materials in a number of biological fluids (5, 6, 8, 9, 12).

The methods currently in use for the killing and removal of bacteria in potential drinking water may or may not also remove pyrogenic substances like endotoxin. Products of preexisting gram-negative bacterial contamination, such as endotoxin, may persist after routine treatment of water. It was noted in this study that if activated carbon columns were used in the water treatment process, measurable endotoxin was found. Such columns have been known to provide a sanctuary for bacterial growth. An increase in the numbers of bacteria during this process would be expected to lead to the persistence of additional free endotoxin, unless somehow removed. The only plant to provide breakpoint chlorination following the carbon filters, the Blue Plains pilot plant, had relatively low endotoxin levels. It is unclear whether this represents removal or destruction of endotoxin or simply prevents further proliferation of endotoxin producing bacteria.

Endotoxin levels from Pomona process 3, where ozonation was practiced, did not differ substantially from the processes where regular chlorination was used. Apparently the final carbon column, after ozonation, permitted bacterial growth and thus endotoxin production.

The two water samples from the Lake Tahoe AWT plant (which had the highest endotoxin content) were inadvertently mailed to Cincinnati instead of being shipped directly to our laboratory in San Antonio. The excessive transportation time without refrigeration undoubtedly allowed proliferation of bacteria and generation of additional endotoxin. Without this mishap, it is possible that the Lake Tahoe samples would have had endotoxin values of the same order of magnitude as the samples from the Pomona and Cincinnati pilot plants.

Miami was the only municipal plant with breakpoint chlorination and was also the only system with no detectable endotoxin. This is consistent with the results from the Blue Plains AWT pilot plant, which showed that endotoxin was low from a system with breakpoint chlorination. Miami was also the only system sampled which used ground water as its source of water supply. Most of the drinking water samples had endotoxin values of the same order of magnitude, within the range of 1.25 to 12.5 ng of endotoxin equivalents per ml. The systems from which these samples were obtained had relatively similar chlorination practices. Overall treatment processes at these plants are related to the quality of the raw water supplies. No particular reason can be given at this time for the relatively high endotoxin values obtained for the Boston and Lawrence samples.

The significance of bacterial endotoxin in drinking water is presently not known. However, the dramatic physiological effects of injecting endotoxin into test animals and man have been widely documented. A recent article on this subject by DiLuzio and Friedmann (4) stated that the presence of endotoxin in drinking water does not seem to constitute a health hazard in normal subjects in whom absorption is limited and adequate defense mechanisms are available for removal and inactivation of endotoxin. If, however, absorption were increased as a result of increased permeability of the gastrointestinal tract, and if this were coupled with a significant impairment in detoxification of endotoxin, a detrimental effect might result. An example might be the alteration of reticuloendothelial function induced by lead or other agents that can also be found in drinking water, which could possibly interact with endotoxin in water to produce an endotoxemia in persons consuming this water.

The results of these experiments confirm the earlier finding of DiLuzio and Friedmann (4) that the *Limulus* assay may easily be applied to the examination of water for the presence of endotoxin. The levels of endotoxin encountered in the present study are somewhat less than those previously reported by DiLuzio and Friedmann (4). However, definition of actual amounts of endotoxin in a given sample may vary with the potency of the amebocyte lysate preparation and the relative activity of the endotoxin standard used for comparison. Therefore, a partial explanation for the lower levels of endotoxin encountered in the present study may be the use of different lysate and endotoxin preparations. Furthermore, water samples in the study of DiLuzio and Friedmann were not refrigerated during or prior to shipment to his laboratory. Thus, the endotoxin content might be expected to increase during shipment due to proliferation of coliforms present in the samples.

A valid criticism of this pilot study would be that provisions were not made to insure that all water samples remained frozen or even at refrigeration temperature until reaching the laboratory. Thus, some samples achieved relatively warm temperatures prior to arrival in our laboratory. This is a possible explanation for the degree of variability in endotoxin content of the samples tested. For example, the two water samples demonstrating the highest endotoxin levels were the Lake Tahoe samples, which were delayed in shipment.

A further shortcoming of the present study is that determinations of the numbers of viable bacteria per milliliter in the water samples were not performed. A future study should be conducted to determine what portion of the measurable endotoxin in drinking water is attributable to bound endotoxin associated with viable coliforms. Previous research (9) has shown that naturally occurring endotoxin may be either in the form of bound endotoxin (endotoxin remaining in association with the cell wall of viable bacteria) or free endotoxin (endotoxin that has been solubilized without autolysis or disruption of the cells [9]). Bound and free endotoxin may be easily distinguished by comparison of endotoxin levels in a fluid before and after passage through a membrane filter (9).

We have also previously shown that measurement of bound endotoxin in a fluid can be used as a means of quantitating the number of bacteria present in a fluid. Experimentally, a linear relationship exists between the number of cells and the amount of bound endotoxin present over a range of 10^3 to 10^6 bacteria per ml (5). However, when this method was applied to the estimation of numbers of bacteria per milliliter in urine, it was shown to have its greatest predictive value only if greater than 10^5 bacterial per ml were present (5, 6). Similar data are not available concerning the predictive value of the *Limulus* assay for detection of viable gram-negative bacteria in less complex fluids such as water. However, an increase in sensitivity would be necessary to make the *Limulus* test an acceptable substitute for the current coliform and standard plate count determinations on potable water.

This study has shown that the *Limulus* amebocyte lysate test can be easily and relatively inexpensively applied to the examination of potential and actual drinking water. It further has shown that a commercial *Limulus* amebocyte lysate, Difco Pyrotest, is immediately available for use in such studies. If Pyrotest were purchased at its current retail price, the cost per test would be as little as $2.50, which is a fraction of the expense of performing rabbit pyrogenicity tests. Additional advantages of the *Limulus* assay are rapidity (a total test time of less than 2 h), and the fact that no specialized equipment or facilities for maintaining laboratory animals are required. The *Limulus* assay can be performed without difficulty in any microbiology laboratory after a brief period of specialized instruction.

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